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BIOLOGICAL BULLETIN

STUDIES ON SEX-DETERMINATION IN AMPHIBIANS. II.

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It is still an open question whether external factors have any influence on the determination of sex. Much of the experimental work that has seemed to show that sex can be influenced by nutrition, temperature, etc., has either been carried out with too small a number of individuals or the methods used in the experiments have been too crude to give results that could be considered as decisive. It is seemingly quite within the power of the modern investigator to determine definitely whether sex can be influenced by external factors; and careful work, such as that which has been done by Cuénot and Hertwig, should settle this question for all time. If it is found that external factors do influence sex then the current chromosome-sex theory will have to be abandoned or considerably modified; if, on the other hand, it is shown convincingly that external factors have no influence on sex-determination, then the way is cleared for the theory that best explains the facts known to us and offers the most favorable hypothesis for future investigations along this line.

Several years ago I started a series of experiments on the eggs of the common American toad, *Bufo lentiginosus*, in order to study the problem of sex-determination in this amphibian which seems to furnish peculiarly favorable material for an investigation of this kind. In the first experiments that were made the influence of nutrition on the determination of sex was investigated. The results of this work, as shown in a previous paper (King, '07), seem to indicate that neither the quantity nor the quality of the food given the larvæ has any influence on sex. The present paper records the results of the second series of experiments that

was made to study the determination of sex in *Bufo*. The work was carried on in the vivarium of the University of Pennsylvania where the splendid facilities at my command permitted the use of relatively large numbers of individuals in each experiment and the rearing of them under exceptionally favorable conditions.

In all of the experiments in this series the tadpoles were kept in large glass aquaria or in cement tanks which were supplied with running water. Each tank contained a sloping bank of sand and gravel and also a quantity of water plants. With the exception of the one experiment in which the influence of starvation on the determination of sex was being studied, all of the tadpoles used in these investigations received similar food, viz., water plants and minute organisms in abundance and occasionally fine pieces of cooked meat or cereal. According to my observations tadpoles of *Bufo* invariably prefer an animal to a vegetable diet and they readily devour the dead bodies of their companions. To this latter fact can be attributed the loss of many individuals during the course of these experiments. Since there is no apparent relation between mortality and sex among tadpoles reared under artificial conditions, as shown by the investigations of Pflüger ('82), and also by my former work, the individuals in which sex was not ascertained are disregarded in considering the results of these experiments, although the total number of individuals with which each experiment started is given in most cases. The methods used in ascertaining the sex of the individuals were similar to those previously employed (King, '07). Whenever possible the bodies of tadpoles that died were preserved and the sex ascertained by means of sections. As there is considerable individual variation in the size and also in the development of the gonads, even after the metamorphosis of the toads, it was necessary to section the genital organs of nearly one fourth of the total number of individuals in order to ascertain their sex.

I. EXPERIMENTS TO DETERMINE THE SEX RATIO OF THE INDIVIDUALS THAT DEVELOP FROM THE EGGS OF THE RIGHT OVARY AND OF THOSE FROM THE LEFT OVARY.

If sex is determined in the ovary, as many investigators believe, it is possible that the individuals developing from the eggs

of one ovary may show a very different sex ratio from that of the individuals produced from the eggs of the other ovary ; or it may be that the eggs of one ovary produce only females and those of the other ovary only males. The following experiments were made to determine this point for *Bufo*. On April 26, 1907, a female which had just begun to deposit her eggs was killed by pithing. The eggs in the right uterus were separated from those in the left uterus and each set was artificially fertilized, sperm from the same male being used for both lots. Six hundred eggs from each ovary were taken for the purposes of the experiment and placed in tanks of equal size. During the course of their development all of the tadpoles were subjected to similar conditions of temperature and they received similar food. The sex of the first 300 individuals in each set to undergo metamorphosis was ascertained with the result shown in the following table :

TABLE I.

	Total Sex Ascertained.	Males.	Females.	Per Cent. of Females.
Right Ovary.....	300	131	169	56.33
Left Ovary	300	128	172	57.33
Total.....	600	259	341	56.83

As shown in the above summary both lots of individuals gave approximately the same sex ratio ; 56.33 per cent. of the individuals from the right ovary and 57.33 per cent. of those from the left ovary being females. This proportion of females is somewhat higher than that obtained in the majority of the experiments and seems to indicate that there is considerable normal variation in the sex ratio of lots of eggs laid by different females.

The above experiment was repeated with the eggs of another toad in the spring of 1908. Owing to an accident, however, all of the tadpoles developing from the eggs of the left ovary were killed and in only 140 of the individuals that had developed from the eggs of the right ovary was sex ascertained. Of this number 64 individuals were males and 76, or 54.2 per cent., were females.

From the results obtained in these two experiments it is evident that the sex ratio of the individuals produced from the eggs of the right ovary is not materially different from that of the indi-

viduals which come from the eggs of the left ovary. Whatever the factors may be which normally determine sex in *Bufo* they appear to have a similar action on the eggs of both ovaries.

II. THE INFLUENCE OF STARVATION ON SEX-DETERMINATION IN *BUFO*.

During the course of my former experiments an attempt was made to ascertain whether a scarcity of food tends to produce a relatively greater number of males, as several investigators have maintained. The mortality among the young tadpoles was so great, however, that the experiment was abandoned for the time. In the spring of 1907 this experiment was repeated on a much larger scale. Eight hundred eggs which had been laid and normally fertilized in the laboratory on the morning of March 30, were placed in a large tank containing only water and clean sand. No food of any kind was given the tadpoles at first and, as might be expected, they began dying in great numbers about two weeks after hatching. Until the beginning of May the only food that the larvæ obtained was the dead bodies of their companions which were devoured before they could be removed. The tadpoles that lived developed very slowly, and in order to prevent all of them from dying they were given a small amount of meat on the second of May and once a week thereafter. Of the 800 individuals with which the experiment started only 59 lived until their sex could be ascertained. Of this number 24 were males and 35, or 59.32 per cent., were females. This number of individuals is, of course, too small to be of much value for statistical purposes, yet the results of the experiment, as far as they go, seem to indicate that scarcity of food has no influence whatever on sex-determination in *Bufo*. Since the only food that the tadpoles received was animal food it might be inferred, from the rather large proportion of females that were obtained, that nitrogenous food favors the production of females, as Yung ('85) has maintained. The excess of females does not seem to me sufficiently large, however, to warrant such a conclusion. Moreover my previous experiments showed that in a total of 464 individuals that received an exclusive meat diet only 246, or 53.01 per cent., were females, thus making it highly improbable that nitrogenous food has any influence on the determination of sex in *Bufo*.

III. THE INFLUENCE OF THE RIPENESS OF THE EGG AT THE TIME OF FERTILIZATION ON SEX-DETERMINATION IN BUFO.

For several years past Hertwig ('05-'07) has been investigating the problem of sex-determination, principally in various species of *Rana*, and he has already published three contributions to the literature on this subject. In his first paper, which appeared in 1905, Hertwig ('05) suggests that the sexual character of an egg changes during the course of its development in the ovary. In the early stages of its ripening the egg tends to produce a male; in the middle phase of its ripening its tendency is towards the production of a female; and in later phases, when the egg is overripe, it again shows a tendency to develop into a male. This change in the sexual character of the egg Hertwig ascribes in his second paper ('06) to "einem verschiedenen Wechselverhältnis ihrer Hauptbestandteile, der Kernsubstanz und des Protoplasma. . . . Eier, welche relativ ärmer an Kernsubstanz sind, Weibchen liefern, chromatinreichere dagegen Männchen." In his latest communication Hertwig ('07) lays little emphasis on the second part of his theory, but he seems to consider that the results of his experiments warrant the conclusion that the ripeness of an egg at the moment of its fertilization determines its sex. In explaining the results of a certain set of experiments in which the eggs of a female from one locality were fertilized with sperm from a male taken from a different locality, Hertwig further modifies his theory by the suggestion that in some cases the spermatozoön exerts a definite influence on sex-determination, although, as a rule, it is probable that "die Eier zur Zeit der Befruchtung sexuell in so hohem Grad determiniert sind, dass der relativ geringe Einfluss des Samens gar nicht zur Geltung kommen würde."

Morgan ('08) has recently given a brief review of this work of Hertwig's on sex-determination and also a criticism of the results. He states that "Hertwig's attempt to connect his view with the ratio of nucleus to cell-plasm of the egg at different periods of its maturation can hardly be looked upon favorably, since in the frog's egg the nucleus as such has already disappeared when the egg leaves the ovary. The chromosomes are thereafter arranged on the equatorial plate of the first polar spindle. It is, however,

during this period that the degree of ripening is supposed to determine the sex of the egg." Morgan suggests that the results of Hertwig's later experiments seem to show that the male is responsible for sex-determination. The preponderance of one sex over the other would be explicable on the assumption that "more sperm of one kind, if two kinds exist, are injured or that internal processes may lead to the production of more functional sperm of one sex." It is unfortunate for this suggestion of Morgan's that the numerous investigations which have been made of the spermatogenesis of various species of amphibians have so far failed to show the slightest evidence of a dimorphism in the spermatozoa.

The frog offers much more favorable material than the toad for an investigation of the influence of the ripeness of the egg at the time of fertilization on sex-determination, since it is possible to extend the laying period of a frog over several days while the toad lays all of her eggs within a few hours whether she is separated from the male or not. Two different experiments were made with the eggs of *Bufo* in order to study this problem. In the first experiment a pair of toads were used that had been brought into the laboratory early in the morning of March 30, 1907. The female commenced laying at 10.45 A. M. of the same day. At 11.15 A. M. the toads were disturbed and the eggs already laid (Lot *A*) were removed to a jar of fresh water. The female began laying again at 12.05 P. M. and a second set of eggs (Lot *B*) were removed at 12.25 P. M. The third laying (Lot *C*) began about 1 P. M. and was interrupted at 1.15 P. M.; while the fourth and last lot of eggs (Lot *D*) were deposited between 2.15 P. M. and 3 P. M. The laying period for this female, therefore, extended over about four hours. Four hundred eggs were taken from each lot for the purposes of the experiment, each set of eggs being put in a compartment of a large aquarium. The compartments were all approximately of the same size and each contained about the same amount of water and of plant food. All of the 1,600 individuals used in this experiment were, therefore, subjected to similar external conditions during the period of their development. There was no appreciable difference in the rate of growth of the tadpoles in the dif-

ferent compartments and the mortality was nearly the same in each lot. The results of this experiment are summarized in the following table.

TABLE II.

	Total Number Individuals.	Total Sex Ascertained.	Males.	Females.	Per Cent. of Females.
Lot <i>A</i>	400	149	70	79	53.02
Lot <i>B</i>	400	202	88	114	56.43
Lot <i>C</i>	400	133	56	77	57.89
Lot <i>D</i>	400	167	78	89	53.29
Total.....	1,600	651	292	359	55.14

The results of this experiment show a striking uniformity since in all four lots about the same proportion of the individuals in which sex was ascertained were females. The excess of females was slightly greater in the two middle lots (*B* and *C*) than in Lots *A* and *D*. This result would seem to support Hertwig's contention that eggs in the middle phase of ripening at the time of fertilization tend to produce a greater proportion of females. The extreme lots of the series (*A* and *D*) did not, however, give a correspondingly greater proportion of males as the theory demands. It is very probable, therefore, that the slightly greater proportion of females in the two middle lots has no especial significance. The comparatively short interval between the laying of the eggs in Lot *A* and of those in Lot *D* makes no direct comparison possible between the results of this experiment and those of Hertwig on *Rana*, since in some of Hertwig's experiments there was an interval of two or three days between the laying of the different lots of eggs by the same female. Normally the eggs of the frog, as well as those of the toad, are all laid within a short period of time. It would seem, therefore, as if in the above experiment a relatively greater number of males should have developed from the eggs of Lots *A* and *D* if, as Hertwig believes, the ripeness of an egg at the time of fertilization has any influence on its sex and if eggs fertilized in early or in late phases of ripening have a tendency towards male production.

All of the eggs used in this experiment should have been peculiarly favorable for the production of males according to Hertwig's theory, since, owing to the very early spring in 1907,

the eggs were laid fully one week earlier than is the usual breeding time for toads in eastern Pennsylvania. Of the 651 individuals in which sex was ascertained 359, or 55.14 per cent., were females. This result does not accord with Hertwig's view that the younger the egg at the time of its fertilization the greater its tendency towards male production. No eggs were obtained in the spring of 1907 from the end of March until the last week in April when several pairs of toads were brought into the laboratory and a large number of eggs laid and normally fertilized. According to Hertwig's theory these eggs were all overripe and should, therefore, have given a preponderance of males. To this series belong the eggs used in the experiment described in section 1. The results of the experiment in question, which are summarized in Table I., show that in the total of 600 individuals in which sex was ascertained there were 341 or 56.83 per cent. of females. This is about the proportion of females that is apparently normal for the species under natural conditions, and this normal sex ratio does not seem to be altered whether the eggs are laid the latter part of March or retained by the female until the end of April.

The second experiment that was made to test the influence of the ripeness of the egg at the time of fertilization on sex-determination was carried out as follows: A female which was isolated on the afternoon of April 5, 1908, began laying at 2 A. M. the following morning. She was at once killed by pithing and the body, unopened, placed in a moist chamber. At 9 A. M., seven hours later, the eggs were removed from the body of the female and artificially fertilized, sperm from two males being used. Although the eggs appeared perfectly good many of them did not fertilize, and the segmentation of the fertilized eggs was slow and in many cases abnormal. It is evident, from this fact, that post-mortem changes had already begun in the eggs at the time that they were fertilized and that it would not be possible to obtain normal embryos from eggs of *Bufo* that remained unfertilized much longer than seven hours after the death of the female. Although the eggs in this series were placed under the most favorable conditions possible many of them failed to gastrulate and the tadpoles that lived developed very slowly, large numbers of

them dying in the early stages of their development. Of the thousand or more eggs with which the experiment started only 372 tadpoles lived until it was possible to ascertain their sex, and in fully one half of this number the gonads had to be sectioned in order to make sure of the sex. Of the 372 individuals in which sex was ascertained 178 were males and 194, or 52.15 per cent., were females. The sex ratio found in these individuals which developed from "overripe" eggs agrees essentially with that found among the individuals in the first experiment where presumably the eggs were in very early phases of ripening at the time that they were fertilized. In both of these experiments an excess of females was produced, although, to accord with Hertwig's theory, there should have been an excess of males in each case.

The results of these experiments seem to indicate that in *Bufo* sex is not determined by the ripeness of the egg at the time that it is fertilized. Eggs fertilized normally within a period of four hours or artificially seven hours after the death of the female, eggs laid the last of March as well as those laid near the end of April, all produce a slight excess of females and thus give a sex ratio that is practically the same as that found among young toads that have recently completed their metamorphosis under natural conditions.

In connection with these experiments reference may be made to a theory of sex-determination recently elaborated by a physician, Dr. T. E. Reed ('07). Starting with the supposition that the ovum is hermaphroditic and that sex, which is a property or function of the ovum, is determined at the time of the fertilization of the egg, Reed then assumes that there is a sex cycle in the germ-plasm and that "this rhythm extends over a period of twelve hours, six being active or masculine, and six passive or feminine, and changes from day to day as do the tides. . . . The sex of the embryo is determined simply by the period through which the germ plasm happens to be passing when fertilization takes place." According to this theory if an egg is fertilized during the positive period, *i. e.*, when the tide is rising, the resultant embryo is always a male; if fertilization takes place during the negative period, when the tide is ebbing, the embryo is neces-

sarily a female. Reed gives no statistics regarding the working out of this hypothesis, but he states that it holds good for a score or more of cases in man and a number of cases in the breeding of horses and cattle. He does not explain, however, how the theory works in the case of such animals as dogs, pigs, rats, etc., which produce a number of offspring of both sexes at one birth. It can hardly be supposed that the fertilization of the ova in all of these animals invariably takes place "at the turn of the tide" when, according to this theory it might be possible for some of the ova to be fertilized during the positive (male) period and others during the negative (female) period.

An opportunity was offered to test this theory for *Bufo* with the lot of eggs used for the first experiment described in this section. On March 30, 1907, the tide was high at Philadelphia, where the experiments were being carried on, at 2.22 P. M. According to Reed's theory the six hours previous to this time would form the positive period during which all eggs fertilized should develop into males. Of the eggs used for the experiment in question Lots *A*, *B* and *C* were laid and fertilized within the positive period and should, therefore, all have become males. Lot *D*, which was laid about the time of the turning of the tide, might be supposed to produce some females. This last lot, as a matter of fact, did not produce as great a proportion of females as did Lot *B*, which was laid at the middle of the positive period and therefore, according to Reed, at the most favorable time for the production of males. Results similar to these were obtained with another lot of eggs laid within the positive period. Reed's hypothesis, therefore, is not adequate to explain the determination of sex in *Bufo* however valuable it may prove to be as a theory of sex-determination in some of the higher mammals.

IV. THE INFLUENCE OF TEMPERATURE ON THE DETERMINATION OF SEX IN BUFO.

It has been held by several investigators that temperature has a decided influence on sex-determination ; a high temperature favoring the development of females, a low temperature tending towards male production. Most of the experiments which have seemed to give support to this hypothesis have been carried out with the

lower forms, such as *Daphnia* and *Hydatina*. The only experiments which have seemed to show that temperature has an influence on sex-determination in the vertebrates are those recently made by Hertwig on *Rana*.

In the first experiment which he made to study the influence of temperature on sex-determination Hertwig divided a bunch of eggs of *Rana temporaria* into two lots; one lot of eggs was allowed to develop at a temperature of 25° C., the other lot developed at a temperature of 13° C. The mortality among the tadpoles that were subjected to the higher temperature was exceedingly great. Out of a total of 200 individuals only 67 underwent metamorphosis, and of this number 4 were males and 63 females. The individuals placed in the cold water lived much better than the others, although the development of their genital organs was so greatly retarded that their sex could not be ascertained even at the time of metamorphosis. In a second series of experiments on the eggs of *Rana esculenta* Hertwig obtained the result shown in the following table.

TABLE III.

Water Temperature.	Males.	Females.	Per Cent. of Females.
22-30° C.	245	127	34.13
13-21° C.	282	54	16.07

Hertwig considers that the results of these two sets of experiments indicate that a high temperature favors the development of females, although he states that a number of individuals in which sex was ascertained is too small to give a decisive answer to the question. In the second experiment the females formed but 34.13 per cent. of the individuals that had developed in the warm water and but 16.07 per cent. of those that had been reared in the cold water. As this experiment stands it hardly seems to me to offer any evidence in support of the view that a high temperature favors the development of females. Both lots of eggs gave a large proportion of males and normally, according to the investigations of von Griesheim ('81) and of Pflüger, there appears to be an excess of females among both young and adult frogs. It would be interesting to know under what conditions the eggs used in

this second experiment were laid as this knowledge might help to explain the reason for the extraordinary excess of males.

Hertwig repeated this experiment in 1906 using eggs of *Rana temporaria*. None of the tadpoles developing at a temperature of 20° C. lived to undergo metamorphosis and therefore their sex could not be ascertained. Out of 340 larvæ developing at a temperature of 10° C., 74 lived to undergo metamorphosis. Of this number 38 were males, 23 females, and in 11 the sex was not ascertained although the individuals were probably males. Hertwig's conclusion from this experiment is that cold favors the development of males as heat favors the development of females.

The following series of experiments was made to study the influence of temperature on the determination of sex in *Bufo*. Five hundred eggs laid and normally fertilized in the laboratory on the morning of March 31, 1907, were divided into two lots: one lot of 250 eggs was placed in a greenhouse, heated by steam, where the temperature of the water in which the tadpoles were developing varied from 23°–30° C.; the remaining 250 eggs were placed in a tank in an unheated basement room where the temperature of the water in which the tadpoles lived ranged from 14°–18° C. during the daytime and was probably much colder at night. Both lots of tadpoles were given similar food and all of the conditions, excepting the temperature, were made as nearly alike as possible. For convenience in description this set of 500 eggs will be called Lot A. An unusually high temperature has apparently as injurious an effect on the eggs of *Bufo* as it has on the eggs of *Rana* since a great many of the tadpoles that were kept in water with a temperature of 23°–30° C. died before it was possible to ascertain their sex. The tadpoles that lived developed very rapidly and many of them had completed their metamorphosis by the first of June. The tadpoles kept in the cold water were vigorous and healthy, and comparatively few of them died. Their development was very slow, however, as compared with that of the individuals living in the warm water. At the end of May the largest of the tadpoles in this lot had a body length of but 8 mm. and their hind legs were still very small. None of these tadpoles underwent metamorphosis until the third week in June.

From a second lot of eggs laid in the laboratory on March 31, 1907, another 500 eggs (Lot *B*) were taken and divided into two sets of 250 eggs each as in the previous experiment. These eggs were placed under conditions similar to those of Lot *A*. The tadpoles in this series behaved in all respects like those in the first series as regards their rate of development and time of metamorphosis. The following table summarizes the results obtained with these two lots of eggs.

TABLE IV.

Water Temperature.	Number Sex Ascertained.	Males.	Females.	Per Cent. of Females
Lot <i>A</i> } 22-30° C.....	63	19	44	69.84
Lot <i>B</i> }	39	30	9	23.07
Lot <i>A</i> } 14-18° C.....	169	68	101	61.58
Lot <i>B</i> }	117	73	44	37.60

As shown in the above table the sex ratio of the individuals used in these experiments differs considerably from the sex ratio which is presumably normal for the species. The individuals of Lot *A*, whether they had lived in warm water or in cold water, produced an unusually large proportion of females; in the one case 69.84 per cent., in the other 61.58 per cent. of the individuals in which sex was ascertained were females. The most striking deviation from the apparent normal sex ratio is shown in the individuals of Lot *B*. Here the number of females is only 23.07 per cent. in the case of the tadpoles that had been reared in the warm water and 37.60 per cent. among the individuals that were subjected to a temperature of 14°-18° C. during the course of their development. The results of these experiments fail to support Hertwig's contention that a high temperature acting during the course of the development of the tadpoles favors the production of females, while a low temperature tends to produce a relatively greater proportion of males. To be sure one set of eggs (Lot *A*) developing at high temperature gave 69.84 per cent. of females which is a considerably larger proportion of females than is found among young toads under natural conditions; but another set of eggs (Lot *B*) developing under exactly similar conditions produced but 23.07 per

cent. of females. Just as striking a difference is shown in the case of the individuals that were reared in cold water. Lot *A* produced 61.58 per cent. of females, while Lot *B*, developing under similar conditions, gave but 37.60 per cent. of females. The very different sex ratios obtained with lots of eggs subjected to the same temperature conditions seem to indicate that sex in *Bufo* is not determined by the temperature of the water in which the tadpoles develop.

If sex is already determined in the egg and not influenced by external factors there is probably a considerable variation in the sex ratios of lots of eggs laid by different females. It might, therefore, be possible to explain the above results on the assumption that, by chance, batches of eggs were taken for the experiment which gave the extremes of possible normal variations in the sex ratio. This explanation is hardly a satisfactory one, especially in light of the results obtained in the experiment about to be described. The batch of eggs from which the individuals used in Lot *A* were taken was laid in water which ranged from 16°–18° C. during the two hours or more in which the eggs were being deposited and fertilized. The eggs used in Lot *B*, on the other hand, were laid in water which had a temperature of from 11°–13° C. during the period of their deposition. The results of the experiment at once suggested the idea that the temperature of the water at the time that the eggs were fertilized might possibly have some influence on sex ; a high temperature favoring the development of females, a low temperature that of males. The following experiment was made to determine the value of this suggestion.

On April 5, 1908, a pair of toads were placed in water which remained at a temperature of about 26° C. during the time that the eggs were being deposited and fertilized. All of the eggs that were laid fertilized readily at this temperature and all segmented in a normal manner. For convenience in description this set of eggs will be called Lot *C*. On the same date another pair of toads were placed in water which had a temperature of about 9° C. during the period in which the eggs were being deposited. All of the eggs in this set, which will be called Lot *D*, were also fertilized and all developed normally. There was,

therefore, a difference of about 17° C. in the temperature of the water in which these two sets of eggs were laid. The eggs of each lot, several thousand in number, were put in a large cement tank supplied with running water and containing plenty of food. The individuals in Lot *C* developed somewhat more rapidly than those in Lot *D* and they began their metamorphosis sooner. The experiment had to be discontinued on July 7, although a number of individuals in each set had not yet reached a stage of development when it would be possible to ascertain their sex. The results of the experiment, tabulated according to the weeks when the individuals died or completed their metamorphosis, is given in the following summary.

TABLE V.

Lot C.				Lot D.		
Date.	No. Sex Ascertained.	Males.	Females.	No. Sex Ascertained.	Males.	Females.
May 24-						
June 6.....	126	66	60	34	18	16
June 7-13 ...	328	160	168	132	98	34
June 15-20 ...	790	344	446	808	524	284
June 22-27 ..	697	255	442	924	422	502
June 28-						
July 7	237	128	109	185	119	66
Total.....	2,178	953	1,225	2,083	1,181	902

As shown in the above table the sex was ascertained of 2,178 of the individuals that developed from the eggs laid in warm water. Of this number 1,225, or 56.24 per cent., were females, thus giving a sex ratio for Lot *C* that is little, if any, higher than that which is apparently normal for the species. The results of this part of the experiment, therefore, do not seem to favor the suggestion that a high temperature acting on the eggs at the time of their fertilization tends to produce a relatively greater number of females. There is the possibility, however, that this negative result may be due to the fact that in making the experiment a higher temperature was used than that which is the optimum for the production of the greatest number of females and that many more females would have developed had the eggs been fertilized in water with a temperature but slightly higher than that used in the former experiment with Lot *A*.

The sex ratio found among the individuals in Lot *D*, agreeing with that of Lot *B*, shows a considerable deviation from the sex ratio that is presumably normal for the species. In a total of 2,083 individuals which developed from eggs laid in water with a temperature of 9° C. there were 902 or 43.30 per cent. of females. Only once in the course of my investigations on sex-determination in *Bufo* have I found the proportion of females in a lot of individuals anywhere near as low as that found in Lots *B* and *D* in the above experiments. In studying the influence of nutrition on sex-determination in *Bufo* I found that in a total of 349 individuals that had been fed on mixed animal and vegetable food only 45.84 per cent. were females. Other lots of eggs laid by the same females gave as high as 59 per cent. of females however ; and on summarizing the results obtained in the entire series of experiments it was found that the females formed 53.58 per cent. of the total number of individuals in which sex was ascertained. In the last experiment described there were 2,127 or 49.91 per cent. of females among the 4,261 individuals whose sex was ascertained. This fact strongly suggests that the sex ratio in this lot of individuals was affected by the conditions under which the eggs were laid.

The results of the experiments described in this section indicate, although they by no means prove, that the temperature of the water in which the eggs are fertilized has some influence on the determination of sex in *Bufo*. A high temperature appears to favor the development of females ; a low temperature, on the other hand, seems to lead to the development of relatively more males. It is possible, as before suggested, that the results obtained in these experiments may be due to the fact that the sex ratio in *Bufo* is a variable one, the proportion of females ranging from 35-70 per cent. in lots of eggs laid by different females. If such is the case it surely is a strange coincidence that lots of eggs should have been taken for these temperature experiments which would give the greatest extremes in the sex ratio.

One serious objection that can be made to these experiments is that the eggs which were fertilized in warm water were laid by one female while the eggs fertilized in the cold water were laid by another individual. This gives an opportunity for the results

to be influenced by possible normal variations in the sex ratio in different lots of eggs. The two sets of eggs were fertilized by sperm from different males also and there is the possibility, as Morgan has suggested, that the male is responsible for sex-determination in amphibians. The last experiment was carried out in the manner described in order to obtain as large a number as possible of eggs that were fertilized normally at different temperatures, since it is not safe to draw conclusions from the results of experiments in which comparatively few individuals are used. Another series of experiments will be made next spring which, it is hoped, will determine definitely whether temperature can influence sex in *Bufo*. An account of the experiments already carried out is published in the hope that other investigators working in the same field may consider the suggestion here made as of sufficient value to warrant testing on any suitable material with which they may be working.

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